Research Article

Functional TCR Retrieval from Single Antigen-Specific Human T Cells Reveals Multiple Novel Epitopes

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Abstract

The determination of the epitope specificity of disease-associated T-cell responses is relevant for the development of biomarkers and targeted immunotherapies against cancer, autoimmune, and infectious diseases. The lack of known T-cell epitopes and corresponding T-cell receptors (TCR) for novel antigens hinders the efficient development and monitoring of new therapies. We developed an integrated approach for the systematic retrieval and functional characterization of TCRs from single antigen-reactive T cells that includes the identification of epitope specificity. This is accomplished through the rapid cloning of full-length TCR-α and TCR-β chains directly from single antigen-specific CD8+ or CD4+ T lymphocytes. The functional validation of cloned TCRs is conducted using in vitro–transcribed RNA transfer for expression of TCRs in T cells and HLA molecules in antigen-presenting cells. This method avoids the work and bias associated with repetitive cycles of in vitro T-cell stimulation, and enables fast characterization of antigen-specific T-cell responses. We applied this strategy to viral and tumor-associated antigens (TAA), resulting in the retrieval of 56 unique functional antigen-specific TCRs from human CD8+ and CD4+ T cells (13 specific for CMV pp65, 16 specific for the well-known TAA NY-ESO-1, and 27 for the novel TAA TPTE), which are directed against 39 different epitopes. The proof-of-concept studies with TAAs NY-ESO-1 and TPTE revealed multiple novel TCR specificities. Our approach enables the rational development of immunotherapy strategies by providing antigen-specific TCRs and immunogenic epitopes. Cancer Immunol Res; 2(12); 1230–44. ©2014 AACR.

Introduction

T cells determine the clinical outcome of various diseases, including infections, cancer, and autoimmune diseases, through the recognition of antigenic peptides bound to HLA molecules on the surface of target cells (1). The recognition of HLA-presented α/β T-cell epitopes is exclusively mediated by the highly polymorphic heterodimeric complex of the T-cell receptor (TCR-α and TCR-β chains. Strategies to decipher the fine specificity of antigen-specific T-cell responses against disease-associated antigens and identification of the corresponding TCR sequences on the single-cell level will promote basic research and broaden the scientific basis for the rational development of T-cell-mediated targeted immunotherapies, including vaccination, modulation of aberrant T-cell reactivity associated with autoimmune disease, and adoptive transfer of TCR-engineered T cells.

Antigen-specific T-cell responses are typically directed against a set of selected immunogenic peptides derived from a full-length antigen. The immunogenicity of a peptide is determined by several factors: It must be expressed, properly processed by proteases, efficiently loaded onto HLA molecules, and bind with sufficient affinity to form a stable peptide–HLA complex that is transported to the cell surface. Finally, surface-expressed peptide–HLA complexes must be able to trigger the activation of a T cell with a complementary TCR (2). Immunogenic epitopes for many well-known clinically relevant antigens have been characterized extensively (http://www.cancerimmunity.org/; refs. 3, 4), but there is a growing list of novel antigens for which knowledge of confirmed epitopes is still scarce or lacking.

The presence of epitope-responsive T cells in the peripheral repertoire is dependent on the generation of appropriate TCR-α/β chains upon somatic recombination of V(D)J gene segments during T-cell development, survival of developing T cells...
during positive and negative selection in the thymus, and in the case of antigen-experienced T cells, on successful priming and proliferation of the mature T cells in the lymphatic system (5).

Several methods have been developed to track clonally expanded T-cell populations to identify protective or pathologic TCR clonotypes (6). One approach is the molecular analysis of TCR diversity by analyzing the TCR-α/β chain CDR3 repertoire using CDR3 spectratyping techniques (7). During the last years, several PCR-based methods have been developed, allowing TCR repertoire analyses at the single-cell level (8–12) as well as the in vitro reconstitution of full-length α/β TCRs (13–16) from single cells. Likewise, next-generation sequencing methods have evolved that generate millions of short sequence reads, which enable high-throughput profiling of TCR repertoires but have not addressed single T cells, thus precluding analysis of paired TCR-α/β chains (17–19). Only recently three different approaches addressing the latter limitation were reported. Linnenmann and colleagues (20) exploited the quantitative nature of TCR gene capture for the identification of TCR repertoires, but none of them combines both, matching epitopes with TCR repertoire.

In parallel, many immunomics tools have evolved, including immunoinformatics enabling in silico prediction of epitopes for whole proteomes following in vitro validation of peptide candidates by HLA binding and cellular immune assays (23, 24). In summary, these methods focus on either the identification of T-cell epitopes or the analysis of TCR repertoires, but none of them combines both, matching epitopes with TCR repertoire.

In this report, we introduce an integrated approach for rapid and efficient cloning of TCR-α/β chains directly from single antigen-specific CD8+ or CD4+ T lymphocytes of individual repertoires combined with rapid functional characterization of the cloned TCRs, including definition of recognized epitopes. The technique was applied to identify TCRs and epitopes for the viral model antigen CMV-p65, the well-characterized tumor antigen NY-ESO-1, and the novel tumor antigen TPTE (Transmembrane Phosphatase with TEnsin homology). These proof-of-concept studies demonstrated that our technology enables the examination of antigen-specific TCR repertoires, the isolation of TCR genes for therapeutic or diagnostic use, and the identification of novel T-cell epitopes and epitope-cluster regions for known and novel disease-associated antigens.

Materials and Methods

Cell lines and reagents

The murine embryonal fibroblast cell line NIH-3T3 and the human chronic myeloid leukemia cell line K562 were obtained from the ATCC and cultured under standard conditions. K562 cells transiently or stably transfected with HLA allelotypes (referred to, e.g., as K562-A’0201) were used for validation assays. The Jurkat76 T-cell line, deficient for both TCR-α and TCR-β chains, was kindly provided by Dr. M. Heemskerk (Department of Hematology, University Medical Center, Leiden, the Netherlands; ref. 25) and was also cultured under standard conditions. The monospecific CTL cell line IVSB specific for the HLA-A’0201–restricted tyrosinase-derived epitope tyr368–376 (26) was cultured in AIM-V medium (Invitrogen) with 10% human serum type AB (Lonza), 350 U/mL IL2 (Richter-Helm Biologics), 5 ng/mL IL7 (PeproTech), and 10 ng/mL IL15 (R&D Systems), and was stimulated weekly with irradiated SK29-Mel and AK-EBV cells. The cell lines IVSB, SK29-Mel, and AK-EBV were kindly provided by Prof. Dr. T. Wölfel (Department of Medicine III, Johannes Gutenberg-University, Mainz, Germany). All cell lines were tested and validated to be Mycoplasma free; no other authentication assay was performed.

Serotyping

An ELISA based on crude lysates of bacteria (CrELISA) expressing either full-length NY-ESO-1 or the N-terminus of TPTE (aa 1–51) was conducted as previously described (27). CMV seropositivity was analyzed by a standard ELISA for polyclonal CMV-specific IgG responses used for routine diagnostics.

Single-cell sorting of antigen-specific CD8+ or CD4+ T cells

Flow cytometric sorting of single antigen-specific CD8+ or CD4+ T cells was conducted either directly ex vivo from freshly isolated T cells or PBMCs or after 1 week of antigen-specific expansion. Before sorting, 2 × 106 T cells or PBMCs were stimulated with 3 × 105 autologous dendritic cells (DC) loaded with the peptide pool or transfected with in vitro-transcribed (IVT) RNA encoding the antigen of interest or a control antigen, respectively, for 4 to 15 hours, depending on the stimulation mode. Cells were harvested, treated with a phycoerythrin/allophycocyanin (PE/APC)-conjugated anti-IFNγ antibody, a FITC-conjugated anti-CD8, and an APC- or PE-conjugated anti-CD4 antibody using the IFNγ Secretion Assay Kit (Miltenyi Biotec). Sorting was conducted on a BD FACSaria flow cytometer (BD Biosciences). One double-positive cell (IFNγ/CD8 or IFNγ/CD4) per well was harvested in a 96-well V-bottom plate (Greiner Bio-One) containing NIH3T3 carrier cells, centrifuged at 4°C and stored at −80°C.

PCR amplification and cloning of V(D)J sequences

5'-RACE cDNA (3 μL) generated from mRNA of isolated T cells was subjected to 40 cycles of PCR in the presence of 0.6 μmol/L Vα/Vβ-specific oligo pool, 0.6 μmol/L Cα- or Cβ-oligo, 200 μmol/L dNTP, and 5 U pfu polymerase (cycling conditions: 2 minutes at 95°C, 30 seconds at 94°C, 30 seconds annealing temperature, 1 minute at 72°C, final extension time of 6 minutes at 72°C). PCR products were analyzed using Qiagen’s capillary electrophoresis system. Samples with bands at 400 to 450 bp were size fractioned on agarose gels, and the bands were excised and purified using a Gel Extraction Kit (Qiagen). Sequence analysis was performed to reveal the sequence of the V(D)J domains. DNA was NotI-digested and cloned into IVT vectors containing the appropriate backbone for a complete TCR-α/β chain.
Figure 1. Technology platform for cloning and characterization of antigen-specific TCRs. A, T cells of patients with cancer seropositive for the antigen of interest (1) are stimulated with antigen-loaded autologous DCs (2). IFNγ secreting CD4⁺ or CD8⁺ T cells are isolated by flow cytometry (3). Single cells are harvested in multiwell plates for RNA extraction and 5′-RACE PCR (4). Full-length TCR-α/β variable regions are amplified (5) and cloned into vectors containing TCR-α/β constant region cassettes (6) for IVT. For specificity testing, T cells are cotransfected with IVT RNA encoding the identified TCR-α/β chains (7). Autologous DCs or K562 cells expressing the respective antigen/HLA combination are used as antigen-presenting cells (8). Confirmed TCRs are subjected to further characterization including definition of epitopes (9). B, for flow cytometric verification of TCR expression, Jurkat76 cells electroporated with TCR-α/β chain RNA or without RNA were stained with a pan TCR antibody. C, IVT vectors used for expression of antigens (top), HLA molecules (middle), and TCR-α/β chains (bottom). The vector backbone is derived from the pST1-sec-2pGUTR-A(120)-Sap1 plasmid featuring a T7 site and 3′-modifications for increased RNA stability and translational efficacy [3′-untranslated region of the human β-globin gene (3′pGUTR)]. Vector templates for antigens contain sequence modules [leader peptide (sec), MHC class I trafficking signal (MITD)] to increase presentation of epitopes. TCR V(D)J sequences were inserted upstream of the TCR constant region cassettes. APC, antigen-presenting cells.
Table 1. Study synopsis of all investigated individual repertoires and identified TCRs

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<th>Antigen Donor</th>
<th>Expansion (E)</th>
<th>Presort-stimulation (S)</th>
<th>Unique Functional αβ</th>
<th>TCR</th>
<th>HLA-I/II restrictiona</th>
<th>Recognized region</th>
<th>Referenceb</th>
<th>Frequency of discovery</th>
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<td>CMV-pp65</td>
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<td>S: iDC + pp65 pept.</td>
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<td>4</td>
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<td>(3)</td>
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<td></td>
<td></td>
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<td>aa 495–503</td>
<td>(3)</td>
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<td>aa 495–503</td>
<td>(3)</td>
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<td></td>
<td>TCRCD4-CMV#16</td>
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<td>aa 495–503</td>
<td>(3)</td>
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<tr>
<td></td>
<td>E: CD4 ex vivo</td>
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<td>3</td>
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<td>aa 117–139</td>
<td>NR</td>
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<td>aa 337–359</td>
<td>(36)</td>
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<td>2</td>
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<td>aa 495–503</td>
<td>(3)</td>
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<td>2</td>
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<td>not done</td>
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<td>2</td>
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<td>S: iDC + NY-ESO-1 RNA</td>
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<td>2</td>
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<td>S: iDC + NY-ESO-1 RNA</td>
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(Continued on the following page)
## ELISPOT assay

The ELISPOT used to analyze antigen-specific IFNγ secretion of TCR-engineered T cells was performed as described previously (28).

## Fluidigm analysis

RNA isolation from tissues was conducted using the RNasy Lipid Tissue Mini Kit procedure according to the instructions of the manufacturer (Qiagen). RNA was converted to cDNA by using the SuperScript II Kit (Invitrogen) according to the manufacturer’s instructions. For quantitative RT-PCR (qRT-PCR) analysis using the Fluidigm detection system, the samples and assays were prepared and analyzed according to the ‘Fast Gene Expression Analysis Using EvaGreen on the BioMark or BioMark HD System Fluidigm Advanced Development Protocol 37.’ 96 × 96 Gene Expression Dynamic Array IFCs were loaded using the IFC Controller HX. Chip arrays were analyzed via a Fluidigm BioMark HD system. After normalization to the housekeeping gene HPRT1 (sense 5'-TGA CAC TGG CAA AAC AAT GCA-3'; antisense 5'-GGT CCT TTT CAC TGG AAC AAT GCA-3'), the relative expression of TPTE (sense 5'-GAGTCTACAATCTATGCAGTG-3'; antisense 5'-CCATAGTTCCGTGTCTATCTG-3') was quantified using ΔΔCt calculation. A calibrator of 18.2 corresponding to 30 (maximal number of cycles used in the PCR) minus the mean of the HPRT1 values of the samples was used in this analysis.

## Additional methods

Detailed methodology is described in Supplementary Methods.

## Results

### Design and set up of the TCR retrieval and characterization strategy

To obtain functional antigen-specific TCR molecules from natural T-cell repertoires, we developed a three-step procedure comprising isolation of single antigen-reactive T cells from patients (Fig. 1A, top), cloning of TCR-α/β chains from sorted cells (Fig. 1A, middle), followed by the functional validation of complete TCRs (Fig. 1A, bottom).

For isolation of single antigen-reactive T cells, we selected donors with preexisting immune responses against the selected antigens. The presence of CD8+ and CD4+ T-cell responses against an antigen is known to correlate with measurable titers of circulating IgG antibodies (29, 30). Consequently, we included only PBMCs of donors that had antibody responses against the selected antigens. We isolated single antigen-reactive T cells from the peripheral blood of seropositive donors either ex vivo or after 1 week of antigen-specific expansion. Stimulation before sorting was conducted with autologous DCs either pulsed with a pool of overlapping peptides or transfected with IVT RNA encoding full-length antigens (31, 32). Activated antigen-specific T cells were detected by an IFNγ secretion assay (Fig. 1A, top). We sorted single CD8+/IFNγ+ or CD4+/IFNγ+ T cells by flow cytometry, extracted RNA, generated first-strand cDNA, and performed global PCR amplification by a modified SMART protocol (Fig. 1A, middle; ref. 33). Before amplification of variable regions, we verified the presence and integrity of TCR-encoding cDNA by amplification of a TCR-β chain constant region sequence.

A particular challenge was the amplification of unknown full-length V(D)J regions of TCRs, including leader sequences without prior knowledge of the variable domain subtype. To achieve this, we designed and applied sets of sequence-specific but partially degenerated primers covering all functional Vα and Vβ genes (Supplementary Tables S1, S2A, and S2B).

Amplified V(D)J sequences were then cloned into vectors containing TCR-α/β constant region cassettes providing full-length templates for immediate IVT. The discovery of new (‘unique’) TCRs was confirmed by direct sequencing of the V(D)J amplification products before cloning, because dominant TCR clonotypes were repeatedly retrieved. The frequency of discovery for each clonotype was documented to identify dominant clonotypes.

To assess the surface expression of cloned TCR chains, we transfected the TCR-deficient cell line Jurkat76 (25) with TCR-coding IVT RNA and detected recombinant TCRs by flow cytometry (Fig. 1B). To be independent of patient cells, we conducted immunologic validation of identified TCRs using IVT RNA transfer for rapid expression of TCRs in lymphocytes.

### Table 1. Study synopsis of all investigated individual repertoires and identified TCRs (Cont’d)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Donor</th>
<th>Expansion (E)</th>
<th>Presort-stimulation (S)</th>
<th>Unique Functional TCR</th>
<th>HLA-I/-II restriction*</th>
<th>Recognized region</th>
<th>Referenceb</th>
<th>Frequency of discovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID10</td>
<td>E: PBMC + TPTE pept. S: CD14 + TPTE RNA</td>
<td>6</td>
<td>2</td>
<td>TCRCD4-TPT#87</td>
<td>DRB1*1501</td>
<td>aa 177–195</td>
<td>NR</td>
<td>2/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCRCD4-TPT#91</td>
<td>DRB1*1501</td>
<td>aa 173–187</td>
<td>NR</td>
<td>1/12</td>
</tr>
</tbody>
</table>

Abbreviations: CD14, monocytes; IDC, fast DC; IDC, immature DC; NR, not reported; pept., peptide pool.

*aHLA haplotypes are listed in Supplementary Table S8.
bPrevious reports on epitopes of the respective restriction located in the recognized region.

cTCR recognizes an epitope in combination with more than one HLA allele.
Figure 2. TCRs cloned from CMV-pp65-specific CD4^+ and CD8^+ T cells. A, TCRs cloned from ex vivo isolated CD8^+ T cells of donor ID1. IVSB cells were transfected with TCR-α/β chain RNAs, stimulated with K562-A^+0201 pulsed with pp65495–503, and analyzed by IFNγ-ELISPOT. Negative controls: unrelated peptide SSX-2241–249, an irrelevant TCR cloned from a CMV-seronegative donor. Positive control: the tyrosinase-derived tyr368–376 epitope. B and C, TCRs obtained from donor ID2 prestimulated with the whole pp65 antigen. B, TCR-engineered IVSB cells were tested on autologous iDCs or K562-A^+0201 cells loaded with the pp65 peptide pool, pp65495–503 or pp65 IVT RNA. Negative controls: TPTE peptide pool; unrelated peptide SSX-2241–249; irrelevant TCR. Positive control: tyr368–376. C, HLA restriction was analyzed by testing IVSB cells transgenic for TCRCD8-CMV#1 for recognition of K562-expressing HLA class I alleles of the donor pulsed with pp65 overlapping peptides or without antigen as a control (top). Fine specificity was determined with K562-B^+3501 targets pulsed with individual pp65-derived 15-mer peptides (bottom). D, specific killing of peptide-pulsed HLA-transfected K562 cells by TCR-engineered IVSB cells analyzed by luciferase cytotoxicity assay. Killing through the endogenous receptor was determined with tyr368–376-pulsed targets. Negative control: irrelevant TCR. E, determination of HLA restriction of TCRs from CD4^+ T cells of donor ID1. TCRs were transferred into CD4^+ T cells of a CMV-seronegative donor and tested against K562 target cells expressing single HLA class II alleles loaded with pp65 peptide pool by IFNγ-ELISPOT.
and HLA molecules in antigen-presenting cells (Fig. 1C). For functional validation, TCR-α/β chain IVT RNA was transferred into the tyrosinase-specific IVSB T-cell line (26) or into primary CD8+ or CD4+ T cells from healthy donors. As antigen-presenting cells, either autologous DCs or K562 cells expressing the HLA class I and II molecules of the respective donor either stably or transiently after IVT RNA transfer (Fig. 1C) were applied. TCR function and epitope specificity were evaluated by IFNγ-ELISPOT and luciferase-based killing assays (Fig. 1A, bottom).

Identification of TCRs recognizing HLA class I– and II–restricted epitopes of the viral antigen CMV-pp65

To assess whether the method rewards immunologically relevant TCRs, CMV-pp65 phosphoprotein, the major target of human CMV-specific T-cell responses, was used as a model antigen (34). In HLA-A*0201–positive individuals, the bulk of CMV-specific T-cell responses is directed against epitope pp65495-503, which confers immunoprotection against the virus (3). We presensitized CD8+ T cells of donor ID1, a healthy HLA-A*0201–expressing CMV-seropositive individual, with autologous DCs pulsed with this epitope and sorted for IFNγ+/CD8+ T cells. We isolated and cloned six unique TCRs from single cells. Four of them reprogrammed IVSB cells to recognize specifically pp65495-503–pulsed K562-A*0201 cells, while a control TCR that we cloned randomly from a seronegative peptide pool or pp65 RNA, the latter conserving the HLA-A*0201 targets suggested appropriate signaling via the recombinant TCR and maintenance of specificity of the endogenous TCR. Remarkably, all four TCRs differed in their Vα and Vβ rearrangements (Supplementary Table S3).

To test whether TCRs against the immunodominant peptide pp65495-503 can also be recovered upon stimulation with the whole pp65 antigen, CD8+ T cells of HLA-A*0201–expressing donors ID2, ID3, and ID4 were presensitized with pp65 RNA-transfected DCs followed by sorting for CD8+/IFNγ+ T cells (Supplementary Fig. S4). T cells of donor ID2 and ID3 underwent 1 week of antigen-specific expansion before we started our procedure, whereas cells from donor ID4 were sorted directly ex vivo (Table 1). Six of the 12 cloned unique CD8+ T cells–derived TCRs from these three donors were found to reprogram IVSB cells to recognize DCs loaded with pp65 peptide pool or pp65 RNA, the latter confirming recognition of naturally processed epitopes (Fig. 2B and Table 1). As exemplified for TCRCD8-CMV#4, four TCRs were directed against pp65495-503 in the context of HLA-A*0201. Testing of K562 target cells expressing individual HLA class I alleles of the patient revealed HLA-B*3501 as the restriction element for TCRCD8-CMV#1 (Fig. 2C, top). Analysis of individual 15-mer of the pp65 peptide pool mapped recognition to the region of aa 117–131 of pp65, suggesting its identity with the previously reported HLA-B*3501–restricted epitope pp65123-131 (IPSVINVHHY; Fig. 2C, bottom; ref. 35).

TCRCD8-CMV#1 and TCRCD8-CMV#4 were transferred into IVSB cells to further test their ability to confer specific killing of pp65117-131–pulsed K562-B*3501 and pp65495-503–pulsed K562-A*0201 target cells, respectively. Remarkably, for both TCRs, specific killing was comparable with the killing of cytotoxic T lymphocytes (CTLs) with pp65117-131–pulsed K562-A*0201 mediated by the endogenous TCR at all tested E:T ratios (Fig. 2D).

To evaluate the suitability of the approach for cloning of HLA class II–restricted TCRs from CD8+ T cells, PBMCs of donor ID1 were stimulated ex vivo with a pp65 peptide pool followed by sorting for single CD4+/IFNγ+ T cells. Three of the five cloned TCR-α/β chain pairs were capable of reprogramming autologous CD4+ T cells for specific recognition of autologous monocytes pulsed with pp65 peptides (Table 1). Using pp65 peptide pool-pulsed K562 cells expressing individual HLA class II alleles of the donor as targets, we identified HLA-DRB1*0701 as the restriction element for all three TCRs (Fig. 2E). Single peptide-pulsing localized the epitope of TCRCD4-CMV#1 to aa 117–139 of pp65, in which no HLA-DRB1*0701–restricted T-cell epitope has been reported to date (Table 1). The epitopes of TCRCD4-CMV#3 and CMV#5 were mapped to aa 357–359 of pp65, a region in which a HLA-DRB1*0701–restricted epitope has been described previously (36).

In summary, our single-cell TCR cloning and validation procedure successfully retrieved functional TCRs against a viral model antigen from CD8+ and CD4+ T cells.

Cloning of TCRs directed against HLA class I– and II–restricted epitopes of the tumor-associated antigen NY-ESO-1

As compared with T cells recognizing viral antigens, precursor frequencies of T cells recognizing tumor-associated self-proteins are usually low even in primed seropositive patients. To evaluate the capability of our approach to clone functional TCRs from antigen-specific T-cell populations of low abundance, we used NY-ESO-1, one of the best characterized members of the cancer/germline antigen family (37). In patients with cancer, NY-ESO-1 frequently elicits spontaneous CD4+ and CD8+ T-cell responses (29, 38, 39), whose specificities have been mapped over the last years (40, 41).
Figure 4. TCRs from CD4⁺ T cells directed against the tumor antigen NY-ESO-1. A, flow cytometric sorting of NY-ESO-1-specific CD4⁺ T cells from NSCLC patient ID7 after 1 week of expansion. IFN-γ-secreting T cells were isolated after stimulation with autologous NY-ESO-1 RNA-transfected iDCs. Control: iDCs transfected with eGFP RNA. B, identification of HLA-restricting elements for TCRs isolated from CD4⁺ T cells of donor ID6. TCR-engineered CD4⁺ T cells were analyzed by IFN-γ-ELISPOT for recognition of K562 cells expressing individual HLA class II alleles of the donor and pulsed with NY-ESO-1 peptide pool. Negative controls: HIV-gag peptide pool; irrelevant TCR. C and D, mapping of HLA class II-restricted epitopes recognized by TCRs obtained from patient ID6 and ID7. CD4⁺ T cells transfected with NY-ESO-1-specific TCRs were tested for recognition of K562 cells expressing the appropriate HLA class II allele and pulsed with partially overlapping 15-mers representing the whole NY-ESO-1 protein.
Because of its natural immunogenicity, it is a prototype for immunotherapy strategies, and many clinical trials targeting NY-ESO-1 by either vaccination or transfer of TCR-engineered T cells are ongoing.

We selected non-small cell lung cancer (NSCLC) patient ID5 based on his confirmed autoantibody reactivity against NY-ESO-1. Bulk PBMCs from this patient were pulsed with NY-ESO-1 peptide pool and expanded in vitro for 1 week. Approximately 0.3% specific CD8+ IFN-gamma+ T cells were obtained by exposure to autologous DCs transfected with RNA encoding NY-ESO-1. We identified a total of 16 different TCRs from the sorted single cells (Table 1). As shown for TCRCD8-NY#2 and TCRCD8-NY#5, seven TCRs conferred specific recognition of autologous DCs presenting epitopes endogenously processed after transfer of NY-ESO-1 RNA (Fig. 3A). To determine restriction elements, we tested the reactivity of TCR-transfected IVSB effectors against NY-ESO-1 peptide pool-pulsed K562 cells expressing single HLA class I alleles of the donor (Fig. 3B). Remarkably, epitopes of all seven TCRs localized between aa 85 and 111 (NY-ESO-1 peptides P22 to P25) of the NY-ESO-1 protein (Fig. 3C and D), a hydrophobic region known to undergo efficient proteosomal cleavage giving rise to multiple epitopes with various HLA restrictions (41). To our knowledge, this is the first time that these restriction elements have been associated with this region. By screening serial nonamers (Supplementary Table S5), we narrowed down the HLA-B*3508-restricted epitope of TCRCD8-NY#5, -NY#6, -NY#8, and -NY#15 (all differing in their Va/b usage; Supplementary Table S3) to NY-ESO-192-100 (LAMPFATPM; Fig. 3D).

Activated CD8+ T cells from a healthy donor engineered to express TCRCD8-NY#5 efficiently and specifically lysed autologous DCs pulsed with decreasing amounts of the NY-ESO-192-100 Peptide (Fig. 3E, left). Cytotoxicity was dose dependent with an IC50 at a peptide concentration of 10^-10 mol/L. In analogy, TCRCD8-NY#2-reprogrammed IVSB effectors were also capable of killing peptide-loaded K562 cells transfected with the appropriate restriction element (Fig. 3E, right).

It is noteworthy that transfer of TCRCD8-NY#5 successfully reprogrammed both CD8+ and CD4+ T cells for specific recognition of NY-ESO-1, indicating that this TCR is functionally independent of the CD8 coreceptor (Supplementary Fig. S6).

CD4+ IFN-gamma+ T cells obtained from patients ID6 and ID7 (Fig. 4A) were used to clone a total of nine different NY-ESO-1-specific TCRs (Table 1). Mapping of restriction elements (Fig. 4B) and epitopes (Fig. 4C and D) revealed that almost all specificities of these T-helper cell-derived TCRs are novel (Table 1). Seven of these TCRs recognized epitopes in a peptide stretch comprising aa 117-147 in the context of different HLA
Figure 6. TCRs from tumor antigen TPTE-specific CD4⁺ and CD8⁺ T cells. A, TPTE-specific T cells of patient ID9 after 1 week of expansion. CD8⁺ and CD4⁺ T cells secreting IFNγ in response to TPTE peptide-pulsed fast DCs (fDCs) were sorted by flow cytometry. Control: fDCs pulsed with HIV-gag peptide pool. B, specificity and function of CD8⁺ T cells transfected with TCRCD8-TPT#35 obtained from patient ID10. (Legend continued on the following page.)
class II elements, suggesting a hot spot for T-helper cell epitopes (Fig. 4D).

Notably, nearly all NY-ESO-1--specific TCRs isolated from CD4+ T cells (6 of 7 tested) were also functional in CD8+ T cells, indicating that these TCRs are coreceptor independent as well (Supplementary Fig. S7).

Isolation of TCRs recognizing epitopes derived from cancer/germline gene TPTE

Whereas NY-ESO-1 is known to be highly immunogenic, multiple tumor-associated genes are still not characterized on the level of T-cell epitopes. One example is TPTE, a germline-specific protein that is aberrantly transcribed in human cancers of the liver, the prostate, and the lung (42–44).

We found that TPTE mRNA expression in healthy tissues is in fact confined to the testis, epididymis, and placenta, while transcript amounts were below the quantification limit of qRT-PCR in all other normal tissue specimens (Fig. 5A). TPTE expression was detected in 22 of 111 (20%) lung tumor samples (Fig. 5B).

TPTE-reactive serum autoantibodies have been reported in patients with hepatocellular carcinoma (43). Recognition of TPTE by T cells, however, has not been shown so far. We pulsed bulk PBMCs of 3 TPTE-seropositive patients with NSCLC (ID8 to ID10) with a TPTE peptide pool and expanded them in vitro for 1 week. Expanded TPTE-specific T cells were visualized by IFN-γ secretion assay after presensitization with autologous, antigen-pulsed patient-derived DCs and used for single-cell TCR retrieval (Fig. 6A). We identified 27 functional TPTE-reactive TCRs (Table 1), from which one (TCRCD8-TPT#35) was derived from a CD8+ T cell. We revealed that this TCR recognized an HLA-B*0702–restricted, endogenously processed peptide in the region of aa 185–199 and is capable of mediating lysis of TPTE peptide-pulsed target cells (Fig. 6B). By analyzing serial nonamers covering this region, we determined the novel epitope TPTE188–196 (PRWTHILLRL).

The other 26 TCRs isolated from single-sorted CD4+/IFNγ+ T cells were confirmed to recognize HLA class II–derived epitopes on TPTE peptide-pulsed K562 cells transfected with one of the patient’s restriction elements (Fig. 6C; Table 1; Supplementary Table S3). Fine mapping using single peptide-pulsed HLA allele–expressing K562 target cells disclosed that the epitopes were distributed widely over the TPTE protein sequence (Fig. 6D and E and Table 1).

Discussion

The identification of epitopes that evoke protective or pathologic T-cell responses as well as the genetic composition of antigen- or epitope-specific TCR in immune responses has important implications for pathogenesis studies, diagnosis, therapy development, and monitoring of infections, malignancies, and autoimmune diseases. In this study, we introduce a technology platform that combines the systematic analysis of functional antigen-specific TCR repertoires with the identification of corresponding T-cell epitopes. This is accomplished through rapid and efficient cloning of TCR-α and TCR-β chains directly from single antigen-specific CD8+ or CD4+ T lymphocytes of individual repertoires followed by functional characterization of cloned TCRs via IVT RNA. Notably, our process is not only efficient but also allows cloning and functional immunologic characterization of new TCRs within less than 2 weeks.

By applying our newly designed primer sets, we covered 80% of all known Vα/Vβ gene family members (Supplementary Table S2A and S2B). In contrast with established primer sets for the amplification of TCR CDR3 regions from single T cells (10, 13, 14, 45), our primers amplify full-length variable regions. In the elegant study by Ozawa and colleagues (9), they were able to generate matching TCR α/β cDNAs from approximately 20% of processed single cells using the 5’– RACE technique. Depending on the quality of the patient’s material up to 50% paired TCR chains can be obtained using our method. Several groups observed dual TCR expression (9–12), raising the need for functional analysis. Not only are we able to study dual TCR expression, using this method we also can delineate the different (productively rearranged) TCR-α chains of a single T cell. Compared with other strategies that aim to validate functional TCRs from single T cells (15, 16), our approach includes the directed cloning of amplified variable regions into IVT vectors providing the TCR-α/β constant regions for extremely rapid generation of full-length TCRs for validation assays that include the determination of epitope specificity for any antigen. Although next-generation sequencing–based methods and the use of barcodes allows for identification of potentially hundreds of α/β-TCRs in a single experiment (22), validation of these TCRs still requires subsequent cost-intensive gene synthesis and cloning.

In total, we identified 398 TCRs representing 189 different clonotypes. Among these, 56 TCRs were shown to be antigen-specific corresponding to 30% of uniquely identified TCRs. The number of nonspecific TCRs identified depends on the background IFNγ secretion by nonspecific T cells after restimulation. This background is in part donor-specific but also depends on the target cells that are used for restimulation. Furthermore, the given frequency of antigen-specific T cells influences how good the antigen-specific cells can be separated from bystander T cells. The usage of other activation-induced molecules such as CD137 may increase...
the percentage of antigen-specific TCRs of uniquely identified clonotypes. 

*Ex vivo* as well as *in vitro* expanded T cells stimulated either with defined epitopes or whole antigens were exploitable for the isolation of entire TCR panels from individuals. Polyepitopic stimulation of antigen-specific CD4⁺ as well as CD8⁺ T cells before flow cytomeric single-cell sorting without prior knowledge of T-cell epitopes or the HLA allelotype of the respective donor was achieved by transfecting autologous antigen-presenting cells with IVT RNA encoding the respective antigen. Moreover, IVT RNA with optimized translation efficiency and stability was used for analysis of surface expression and function of recombinant TCRs as well as for expression of HLA molecules in target cells for epitope and restriction mapping. In summary, these technical developments enabled an exceptionally fast, flexible, and robust process for discovery and validation of antigen-specific TCRs with need for minimal amount of patient material.

We established the platform using CMV-pp65 as a model antigen and retrieved 13 different CMV-pp65–specific TCR clonotypes (Table 1). These TCRs isolated from CD4⁺ and CD8⁺ T cells not only recognized known CMV-pp65–derived epitopes, but also one novel epitope. This proves that even in extensively characterized antigens, our platform can still discover novel epitopes and identify corresponding novel TCRs.

The proof-of-concept study with the highly immunogenic tumor antigen NY-ESO-1 resulted in retrieval of 16 NY-ESO-1–specific TCRs from single T cells of 3 different patients with NSCLC, which recognized endogenously processed NY-ESO-1 epitopes. Immunologic validation assays revealed novel and known HLA class I– and II–restricted epitopes, which clustered in previously described immunogenic regions of the NY-ESO-1 protein, and thus confirmed the validity of our study (41, 46, 47).

We then addressed TPTE, a tumor antigen that was not yet described as a target for antigen-specific T cells. First, we confirmed the ectopic expression of TPTE in lung cancer tissues by qRT-PCR. Expression of TPTE mRNA was detected in 20% of lung cancer tissues analyzed, but not in healthy tissues. From 3 TPTE-seropositive patients with NSCLC, TPTE–specific T cells were isolated and a total of 27 TPTE–specific TCRs were cloned from these cells. Immunologic validation assays revealed multiple HLA class I– and II–restricted epitopes distributed over the entire sequence of the antigen. To our knowledge, this is the first report that describes TPTE–specific T-cell responses in patients with cancer. Furthermore, we identified multiple novel HLA class I– and II–restricted TPTE–derived T-cell epitopes.

The effectiveness of the platform that we developed is illustrated by the great number of functional TCRs identified. We isolated 56 TCRs specific for CMV-pp65, NY-ESO-1, and TPTE. Furthermore, upon TCR characterization, we found multiple novel epitopes for a viral antigen, a well-studied tumor antigen, as well as a novel cancer antigen. To assess the biologic relevance of individual epitope comprehensive analyses of epitope-specific T-cell responses in larger groups of patients with cancer sharing at least one HLA allele are necessary.

The provision of characterized TCRs with different tumor antigen specificities and HLA restrictions may fuel the development of therapies based on vaccination or adoptive transfer of TCR-engineered T cells that have recently led to promising results (48–50). This approach can be applied for the isolation of high-affinity TCRs, for example, from *in vitro* primed allo-restricted T cells or from autologous mutation-specific T cells. The latter is particularly interesting as cancer mutanome approaches based on identification of nonsynonymus mutations are emerging for individualized cancer therapy. Furthermore, adaption to liquid handling stations will enable the high throughput that is needed to identify immunodominant epitopes shared by different patients with cancer as well as appropriate lead structures for personalized TCR gene therapy.

In summary, we successfully developed and validated an approach for the rapid cloning and functional testing of antigen-specific TCRs from single T cells with broad applicability for research and development of T cell–based immunotherapies. Most importantly, by applying this technique we have extended the universe of TAA epitopes and TCRs considerably.

**Disclosure of Potential Conflicts of Interest**

P. Simon is Head of Immunoreceptor Validation at BioNTech Cell & Gene Therapies GmbH and has ownership interest in a patent application. T. Omo­koko is a senior scientist at BioNTech Cell & Gene Therapies GmbH and has ownership interest (including patents) in a patent application. A. Breitkreuz is an engineer at BioNTech Cell & Gene Therapies GmbH. O. Türeci is a technician at BioNTech Cell & Gene Therapies GmbH, and has ownership interest in a patent. U. Sahin is the cofounder of BioNTech Cell & Gene Therapies GmbH, reports receiving a commercial research grant from BioNTech Cell & Gene Therapies GmbH, and has ownership interest (including patents) in T80N and BioNTech Cell & Gene Therapies GmbH. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Simon, T.A. Omokoko, A. Breitkreuz, S. Kreiter, A. Konur, C.M. Britten, C. Paret, K. Dhaene, U. Sahin

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